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Original Articles

Parallel Synthesis of Cell-Penetrating Peptide Conjugates of PMO Toward Exon Skipping Enhancement in Duchenne Muscular Dystrophy

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We describe two new methods of parallel chemical synthesis of libraries of peptide conjugates of phosphorodiamidate morpholino oligonucleotide (PMO) cargoes on a scale suitable for cell screening prior to in vivo analysis for therapeutic development. The methods represent an extension of the SELection of PEPtide CONjugates (SELPEPCON) approach previously developed for parallel peptide-peptide nucleic acid (PNA) synthesis. However, these new methods allow for the utilization of commercial PMO as cargo with both *C*- and *N*-termini unfunctionalized. The synthetic methods involve conjugation in solution phase, followed by rapid purification via biotin-streptavidin immobilization and subsequent reductive release into solution, avoiding the need for painstaking high-performance liquid chromatography purifications. The synthesis methods were applied for screening of PMO conjugates of a 16-member library of variants of a 10-residue ApoE peptide, which was suggested for blood-brain barrier crossing. In this work the conjugate library was tested in an exon skipping assay using skeletal mouse *mdx* cells, a model of Duchene's muscular dystrophy where higher activity peptide-PMO conjugates were identified compared with the starting peptide-PMO. The results demonstrate the power of the parallel synthesis methods for increasing the speed of optimization of peptide sequences in conjugates of PMO for therapeutic screening.

Introduction

Duchenne muscular dystrophy (DMD) is a severe muscle-wasting disease in young boys caused by deletions or point mutations in the pre-mRNA for dystrophin that result in out-of-frame transcripts and hence a nonfunctional truncated dystrophin protein. In recent years, synthetic splice-switching oligonucleotides (SSO) have been developed as new treatments for DMD whereby an SSO is targeted to the pre-mRNA and mediates splicing redirection to restore the reading frame of the dystrophin gene via exon skipping, and thus to generate a shorter but functional dystrophin protein isoform [1,2]. Several SSO chemistries have been developed for exon skipping in DMD and other neuromuscular diseases [3,4], but of these only two SSO chemical types have been used in clinical trials, namely 2′-*O*-methyl phosphorothioates (2′-OMe/PS) [5] and phosphorodiamidate morpholino oligonucleotides (PMO) [6,7].

Peptide (P)-PMO conjugates consisting of charge neutral PMO conjugated to cell-penetrating peptides (CPPs) have been designed in order to enhance delivery into cells. The most effective CPPs are Arg-rich [8,9]. P-PMOs have shown significantly improved uptake in an *mdx* mouse model of DMD, where the dystrophin gene has a point mutation in exon 23, both in muscle cell culture and in skeletal muscle following systemic delivery [10–12]. However, good heart activity has typically required repeated and very high dose administrations [13].

Starting from a derivative of the already known CPP penetratin containing six additional arginine residues (R6-Pen) [14], we derived a series of peptide nucleic acid (PNA)/PMO internalization peptides (Pips) with improved stability to serum proteolysis. In collaborative studies with the laboratory of Wood and colleagues, two of these Pip peptides, Pip2a and Pip2b, when conjugated to a dystrophin exon 23–specific peptide nucleic acid (PNA) SSO, showed strong exon skipping and dystrophin production in *mdx* muscle cells and following intramuscular injection into the tibialis anterior (TA) muscle of the *mdx* mouse [15]. Further Pip5 peptide versions as PMO conjugates showed remarkably high dystrophin production in heart in addition to skeletal muscle, following systemic delivery into *mdx* mice [16]. Such Pip peptides are comprised of a hydrophobic core region flanked on each side by an Arg-rich domain containing

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aminohexanoic (X) and β-alanine (B) spacers. In a further Pip6 series of peptides, the hydrophobic core was found to be essential for dystrophin production in heart [17]. Pip6a-PMO in particular has become a paradigm for physiology and other mouse studies in DMD and other neuromuscular diseases.

A significant problem in developing novel CPPs tuned specifically for delivery into different cell types or tissues has proved to be the lack of a rapid method for synthesis of sequence variants of CPPs conjugated to a PMO or PNA cargo on suitable scale for cell culture testing to allow CPP candidates to emerge for subsequent in vivo studies. Recently, we developed a new method, known as SELection of PEPtide CONjugates (SELPEPCON), for the parallel synthesis of peptide-biocargo conjugates that utilizes affinity purification for rapid isolation of the conjugates and which avoids the need for time-consuming high-performance liquid chromatography (HPLC) conjugate purifications [18]. This methodology was applied initially to create a parallel-synthesized CPP library of a PNA cargo specifying the well-known splice-redirecting 705 sequence [19]. A conjugate library of some 78 CPP-PNA members was tested for their abilities to induce splicing redirection in HeLa pLuc705 cells and the results showed proof-of-principle as to how SELPEPCON could be applied for conjugate cell screening purposes.

We now extend this SELPEPCON methodology for use with a PMO cargo. We describe the design and synthesis of a small library of peptide-PMO conjugates through use of a biotinylated azido connector peptide pre-joined to the PMO cargo, which was then conjugated via copper-catalyzed ''click'' chemistry to alkyne-functionalized peptides in the library. After immobilization on streptavidin cartridge columns, the conjugates were released by reductive cleavage of a reversible disulfide linkage, cysteine-capped, and desalted. The conjugate library was tested for exon skipping biological activity using mouse *mdx* muscle cells in culture and this showed substantial peptide sequence dependence. A second method of SELPEPCON peptide-PMO conjugation using direct amide conjugation of a *C*-terminally extended peptide library to the PMO was also described and exemplified by use of an arginine-rich peptide, Pip6a. The methods are applicable in principle for rapid screening of CPP-PMO conjugates with varying peptide sequences for targeting mRNA or pre-mRNA in any disease where PMO can be used for steric blocking antisense activity.

Materials and Methods

9–fluorenylmethoxycarbonyl (Fmoc) protected L-amino acids, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium (PyBOP), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and the Fmoc-β-Ala-OH preloaded Wang resin (0.19 mmol/g) were obtained from Merck. Fmoc-L-bis-homopropargylglycine-OH was obtained from Chiralix. HPLC grade acetonitrile, methanol, and synthesis grade *N*-methyl-2-pyrrolidone (NMP) were from Fisher Scientific. Peptide synthesis grade *N,N*-dimethylformamide (DMF) and diethyl ether were obtained from VWR International). Piperidine and trifluoroacetic acid (TFA) were obtained from Alfa Aesar. H-Rink amide chemmatrix® resin was purchased from PCAS Biomatrix Inc.. TBTA [*tris*-(benzyltriazolylmethyl)amine] was synthesized according to a literature procedure [20]. PMO was purchased from Gene Tools Inc. (Philomath, OR). Chicken embryo extract and horse serum were obtained from Sera Laboratories International Ltd.. Interferon was obtained from Roche Applied Science. All other reagents were obtained from Sigma-Aldrich unless otherwise stated. Matrix-assisted-laser-desorption/ionization-time of flight (MALDI-TOF) mass spectrometry was carried out using a Voyager DE Pro BioSpectrometry workstation. A stock solution of 10 mg/mL of α-cyano-4-hydroxycinnamic acid or sinapinic acid in 60% acetonitrile in water was used as matrix. Mass accuracy for the instrument is ±0.1%.

Azido connector peptide (2)

The solid-supported connector peptide BWXCG (**1**) (B, beta-alanine; X, aminohexanoic acid) (Fig. 1) was synthesized on a 100-μmol scale using a CEM Liberty™ microwave Peptide Synthesizer and Fmoc chemistry following manufacturer's recommendations. After the solid phase peptide synthesis, the solid-supported peptide was removed from the synthesizer and 5-azidopentanoic acid was manually coupled using HBTU/1-hydroxy-7-aza-benzotriazole (HOAt)/diisopropylethylamine (DIPEA) (2.5:2.5:5.0) at room temperature for 15 minutes to give solid supported azido peptide **2**. The azido peptide was released from the solid support by treatment with a cleavage cocktail consisting of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), H_2O, phenol (91.5%:5%:2.5%:1%, 5 mL) for 3 hours at room temperature. After peptide release, excess TFA was removed by a flow of nitrogen. The crude peptide was precipitated by the addition of cold diethyl ether (15 mL) followed by centrifugation at 2,500 rpm for 2 minutes. The crude peptide pellet was washed thrice with cold diethyl ether (3 × 15 mL). The crude peptide was dissolved in 7 mL 25% dimethylsulfoxide (DMSO) in 0.1% TFA and purified by reversed phase (RP)-HPLC using a Phenomenex Jupiter column (21.2 × 250 mm, C18, 10 mm) at a flow rate 10 mL/minutes with the following gradient (A: 0.1% TFA; B: 90% CH_3CN, 0.1% TFA) 0–2 minutes 5% B, 2–35 minutes 5%–60% B, 35–40 minutes 60%–90% B. The fractions containing the desired azido peptide (**3**) were combined and lyophilized to yield the peptide as a white solid (24% yield) (mass expected 673.78; mass obtained 673.20).

Biotinylated azido connector peptide (5)

To a solution of the azido connector peptide (**3**) (6 μmol) in DMSO (200 μL) was added water (600 μL). To this solution, EZ-link HPDP-Biotin (**4**) (800 μL of 15 mM in DMSO) and sodium phosphate buffer pH 7.4 (200 μL of 100 mM) were added and the resulting mixture was left to stand for 2 hours at room temperature. The reaction was quenched by the addition of 0.1% TFA (2 mL). This solution was filtered using a solid phase extraction reservoir (12 mL, Agilent Technologies), fitted with a polypropylene frit (20 μm, Agilent Technologies) and the desired product was isolated using RP-HPLC using a Phenomenex Jupiter column (21.2 × 250 mm, C18, 10 μm) at a flow rate 10 mL/minute with the following gradient (A: 0.1% TFA; B: 90% CH_3CN, 0.1% TFA): 0–30 minutes 10%–70% B, 30–33 minutes 70%–90% B. The fractions containing desired modified peptide were combined and lyophilized to yield the desired biotinylated azido connector peptide **5** as a white solid (78% yield) (mass expected 1,102.40; mass obtained 1,101.32).

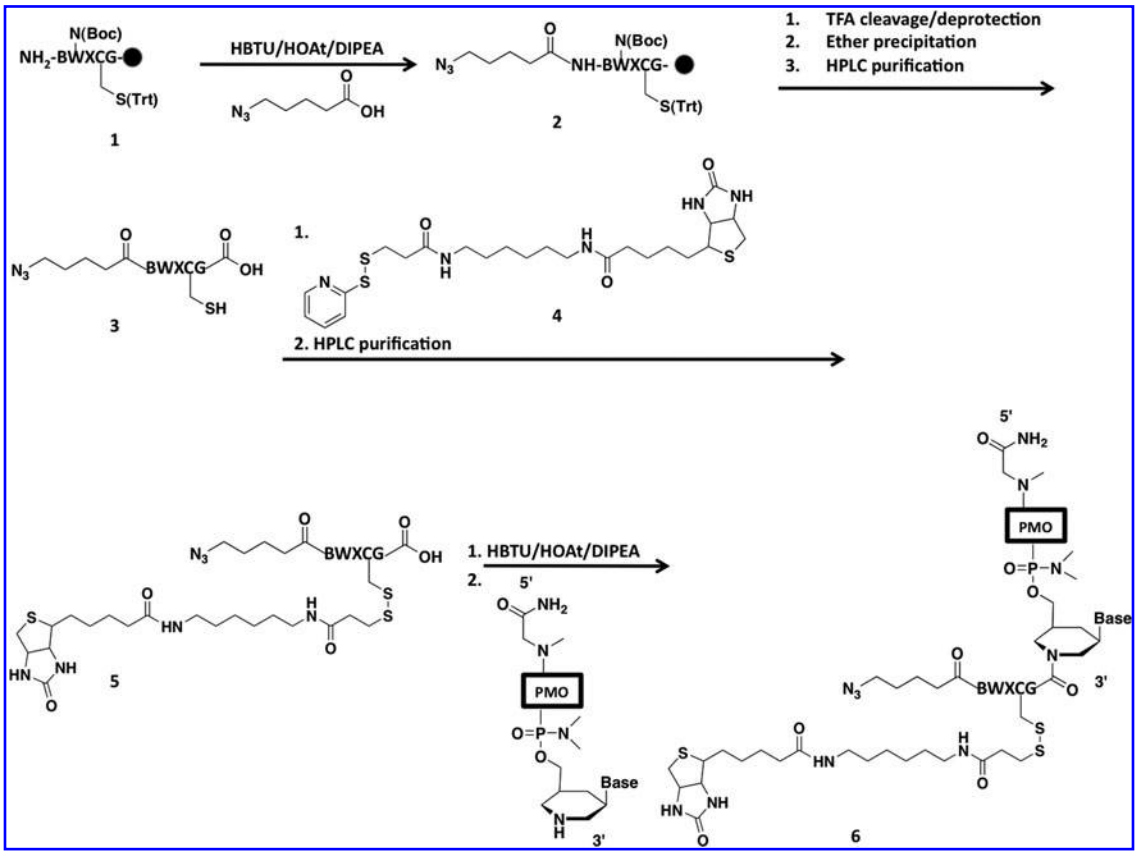

FIG. 1. The synthesis of the biotinylated azido connector peptide and subsequent *C*-terminal amide attachment to a phosphorodiamidate morpholino oligonucleotide (PMO) cargo. DIPEA, diisopropylethylamine; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-aza-benzotriazole; HPLC, high-performance liquid chromatography; N(Boc), N(t-butyloxycarbonyl); S(Trt), S(Trityl); TFA, trifluoroacetic acid.

Biotinylated azido connector peptide derivatized PMO (6)

An unmodified 25-mer PMO antisense sequence for mouse dystrophin exon-23 (GGCCAAAACCTCGGCTTACCTG AAAT) with an unfunctionalized C-terminus was used. The connector peptide was conjugated to the 3′-end of the PMO through its *C*-terminal carboxyl group. To a solution of azide and biotin functionalized peptide (2500 nmol) in *N*-methylpyrrolidone (NMP, 80 µL) were added HBTU (19.2 µL of 0.3 M in NMP, 2.5 eq), HOAt in (16.7 µL of 0.3 M NMP, 2 eq), DIPEA (6.25 µmol, 1.0 µL, 2.5 eq), and PMO (200 µL of 5 mM in DMSO, 0.5 eq). The mixture was left for 2 hours at 40°C and the reaction was quenched by the addition of 0.1% TFA (1 mL). This solution was purified by RP-HPLC using a Phenomenex Jupiter column (4.6×250 mm, C18, 5 µm) with the following gradient (A: 0.1% TFA, B: 90% CH_3CN, 0.1% TFA) 0–30 minutes 10%–60% B, 30–33 minutes 60%–90% B. The fractions containing the desired compound were combined and lyophilized to yield the PMO-connector peptide derivative **6** as a white solid (28% yield) (mass expected 9,497.48; mass obtained 9,490.53).

Preparation of a library of Apolipoprotein E peptide variants

The first amino acid, Fmoc-L-bis-homopropargylglycine-OH, was coupled manually to the solid support (H-Rink Amide Chemmatrix®, 0.53 mmol/g loading) using a 1.5 molar excess following HBTU activation. The remainder of each sequence was prepared on a 5-µmol scale using an Intavis Parallel Peptide Synthesizer by applying standard Fmoc chemistry and following manufacturer's recommendations. Double coupling steps were used with a PyBOP/NMM coupling mixture followed by acetic anhydride capping after each step. The *N*-terminally acetylated peptides were cleaved from the solid support by treatment with a cleavage cocktail consisting of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), H_2O, and phenol (91.5%: 5%: 2.5%: 1%, 1.5 mL) for 3 hours at room temperature. The solutions were concentrated to a volume of 500 µL using a flow of nitrogen and diluted with water (5 mL). The resulting mixture was loaded on a 20-cc Oasis HLB (hydrophilic-lipophilic balance) cartridge (Waters), which was previously washed with acetonitrile (10 mL) and equilibrated with 0.1% TFA (2×10 mL) and 5% acetonitrile in 0.1% TFA (2×10 mL). The peptide was eluted with 40% acetonitrile in 0.1% TFA (10 mL). The solution obtained was lyophilized and the yield was calculated using the weight obtained, corrected for the amount of TFA salts based on the number of positive charges present in the peptide.

Conjugate synthesis by copper-catalyzed click chemistry

A mixture was prepared containing connector peptide-functionalized PMO (30 nmol of a 1 mM solution in water), alkyne functionalized peptide (150 nmol of a 10 mM solution in NMP), and 2,6-lutidine (15,000 nmol). A premixed solution

containing $CuSO_4$-TBTA solution (12 μL of 20 mM, 240 nmol) in a 1:1 mixture of H_2O/DMSO and sodium ascorbate (30 μL of a 20 mM solution in water, 600 nmol) was added to this mixture. The mixture was left for 2 hours and quenched by the addition of ethylenediaminetetraacetic acid (EDTA) (900 μL of 10 mM in TBS 10% CH_3CN).

The resulting solution was loaded in two batches of 500 μL on a streptavidin HP SpinTrap (GE Healthcare) column and incubated for 1 hour. The column was washed with EDTA (400 μL of 10 mM in TBS) and with TBS (5 × 400 μL). The conjugate was released from the resin by 2 × 20 minutes reaction with tris(2-carboxyethyl)phosphine (TCEP) (2 × 400 μL of 10 mM in 20% CH_3CN TBS) and the resin washed with TBS (200 μL). The resulting solutions were combined and lyophilized. The resultant white solid was dissolved in 0.1% TFA, 10% CH_3CN (500 μL) and loaded on an equilibrated 1-cc Oasis HLB cartridge (Waters) together with 0.1% TFA (500 μL). The column was washed with 0.1% TFA (3 × 1 mL), 5% CH_3CN in 0.1% TFA (3 × 1 mL), and 10% CH_3CN in 0.1% TFA (1 × 1 mL). The conjugate was released from cartridge via the addition of 60% CH_3CN in 0.1% TFA (500 μL). The resulting solution was lyophilized to yield the desired conjugate as a white solid (35%–64% yield).

Iodoacetamide capping procedure

Each conjugate (10 nmol) was dissolved in sodium bicarbonate solution (25 μL of 0.1 M, pH 8.0) and iodoacetamide (23 mg, 250 nmol) was added. The solution was left for 2 hours in darkness and diluted with water (975 μL). Excess iodoacetamide was removed through the use of an Amicon® Ultra-1 mL centrifugal filter unit with a 3,000 molecular weight cutoff. The resulting filtrate was lyophilized to yield the desired thiol-capped product (55%).

Alternative capping method of free thiol groups of conjugates (unsuitable for very hydrophobic peptides)

Following the click reaction and subsequent release from the strepavidin HP spin trap columns using TCEP, the unpurified solution was treated with a large excess of iodoacetamide (25 eq, 139 mg, 1,500 nmol). The solution was left for 2 hours in the dark and diluted with water (4 mL). Excess iodoacetamide and TCEP were removed through the use of Amicon® Ultra-5 centrifugal filter units with a 3,000 molecular weight cutoff. The resulting filtrate was lyophilized to yield the desired S-capped product (65%).

Alternative method of conjugate synthesis without precoupling with a connector peptide: Pip6a-SH-PMO

Pip6a-SH (Ac-RXRRBRRXRYQFLIRXRBRXRCXB, where B is β-alanine and X is aminohexanoic acid) was synthesized as a *C*-terminal carboxylic acid on an Fmoc-β-Ala-Wang resin (100–200 mesh, 0.6 mmol/g) using Fmoc chemistry on a CEM Liberty™ microwave peptide synthesizer. The side chain protecting groups used were trifluoroacetic acid labile. The peptide was synthesized on a 0.1 mmol scale using a 5-fold excess of Fmoc-protected amino acids (0.5 mmol), which were activated using PyBOP (5-fold excess) in the presence of DIPEA. Piperidine (20% v/v in DMF) was used to remove the Fmoc protecting groups. The coupling was carried out once at 75°C for 5 minutes at 60-watt microwave power except for arginine residues, which were coupled twice each. Each deprotection reaction was carried out at 75°C twice, once for 30 seconds and then for 3 minutes at 35-watt microwave power.

Once synthesis was complete, the support was washed with DMF (3 × 50 mL) and the *N*-terminus of the solid phase bound peptide was acetylated with acetic anhydride in the presence of DIPEA. The peptide was cleaved from the solid support by treatment with a cleavage cocktail consisting of trifluoroacetic acid (TFA), 3,6-dioxa-1,8-octanedithiol, H_2O, triisopropylsilane (TIPS) (94%:2.5%:2.5%:1%, 10 mL) for 3 hours at room temperature. Excess TFA was removed by a flow of nitrogen. The cleaved peptide was precipitated via the addition of ice-cold diethyl ether and centrifuged at 3,000 rpm for 2 minutes. The peptide pellet was washed in ice-cold diethyl ether thrice. The crude peptide was dissolved in water, analyzed and purified by RP-HPLC on Phenomenex Jupiter columns (analytical: 4.6 × 250 mm, C18, 5 μm) and (preparative: 21.2 × 250 mm, C18, 10 μm) respectively. A flow rate of 1.5 mL/minute for the analytical column and 10 mL/minute for the preparative column with the following gradient (A: 0.1% TFA, B: 90% CH_3CN, 0.1% TFA) 0–2 minutes 5% B, 2–35 minutes 5%–60% B, 35–40 minutes 60%–90% B was used. The fractions containing the desired peptide were combined and lyophilized to give the product as a white solid (55% yield) (mass expected 3,169; mass obtained 3166).

Biotinylation of Pip6a-SH

To a solution of of Pip6a-SH (2 μmol) in DMSO (66 μL) was added water (200 μL). To this solution EZ-link HPDP-Biotin (267 μL of 15 mM in DMSO) and sodium phosphate buffer pH 7.4 (66 μL of a 100 mM solution) were added and the resulting mixture was left to stand for 2 hours at room temperature. The reaction was quenched by the addition of 0.1% TFA (0.66 mL). This solution was filtered using a solid-phase extraction reservoir (12 mL, Agilent Technologies), fitted with a polypropylene frit (20 μm, Agilent Technologies) and the desired product was isolated using RP-HPLC, a Phenomenex Jupiter column (21.2 × 250 mm, C18, 10 μm) was used at a flow rate 10 mL/minute with the following gradient (A: 0.1% TFA, B: 90% CH_3CN, 0.1% TFA) 0–30 minutes 10%–70% B, 30–33 minutes 70%–90% B. The fractions containing the desired peptide were combined and freeze-dried to yield biotin-modified Pip6a-SH as a white solid (48% yield). Mass expected 3597.32, mass obtained 3594.32.

Conjugation of biotinylated Pip6a-SH to PMO and subsequent capping

An unmodified 25-mer PMO antisense sequence targeting mouse dystrophin exon-23 (GGCCAAAACCTCGGCTTACCTGAAAT) with an unfunctionalized *C*-terminus was purchased from Gene Tools Inc.. The biotinylated Pip6a-SH was conjugated to the 3′-end of the PMO through its *C*-terminal carboxyl group. To a solution of biotin-functionalized peptide (625 nmol) in *N*-methylpyrrolidone (NMP, 20 μL) were added HBTU (4.8 μL of 0.3 M in NMP, 2.5 eq), HOAt (4.2 μL of 0.3 M NMP, 2 eq), DIPEA (0.25 μL, 1.56 μmol, 2.5 eq), and PMO (50 μL of 5 mM in DMSO, 0.5 eq). The reaction was carried out at 40°C for 2 hours and

quenched with TBS in 10% CH_3CN. The resulting solution was loaded in two batches of 500 μL on a streptavidin HP SpinTrap column (GE Healthcare) and incubated for 1 hour. The column was washed with EDTA (400 μL of 10 mM in TBS) and with TBS (5 × 400 μL). The conjugate was released from the resin by 2 × 20-minute reaction with TCEP (2 × 400 μL of 10 mM in 20% CH_3CN in TBS) and the resin washed with TBS (200 μL).

The resulting solutions were combined and lyophilized. The resultant white solid was dissolved in 0.1% TFA, 10% CH_3CN (500 μL) and loaded on to an equilibrated 1-cc Oasis HLB cartridge (Waters) together with 0.1% TFA (500 μL). The column was washed with 0.1% TFA (3 × 1 mL), 5% CH_3CN in 0.1% TFA (3 × 1 mL) and 10% CH_3CN in 0.1% TFA (1 × 1 mL). The conjugate was released from the column via the addition of 60% CH_3CN in 0.1% TFA (500 μL). The resulting solution was lyophilized to yield the desired conjugate as a white solid (42% yield).

Conjugates (10 nmol) were dissolved in sodium bicarbonate solution (25 μL of 0.1 M, pH 8.0), and iodoacetamide (23 mg, 250 nmol) was added. The solution was left for 2 hours in the dark and diluted with water (975 μL). Excess iodoacetamide was removed through the use of Amicon® Ultra 1-mL centrifugal filter unit with a 3,000 molecular weight cut off. The resulting filtrate was lyophilized to yield the desired S-capped product (55%) [mass expected 11,622; mass obtained 11,645 (mass + Na)].

Cell culture

Murine H2k *mdx* myoblasts were cultured in gelatin (0.01%)-coated flasks at 33°C, under 10% CO_2 in Dulbecco's modified Eagles medium (DMEM;, PAA laboratories) supplemented with 20% heat-inactivated fetal bovine serum (FBS Gold, PAA Laboratories), 2% chicken embryo extract (Seralab), 1% penicillin-streptomycin-neomycin antibiotic mixture (PSN, Gibco), and 3 pg/μL γ-interferon (Roche).

Cells were seeded in gelatin (0.01%)-coated 48-well plates at a density of 2×10^5 cell/mL and left for 2 days at 33°C, 10% CO_2. To differentiate into myotubes, cells were further grown in DMEM supplemented with 5% horse serum (Sigma) and 1% PSN at 37°C under 5% CO_2 for 2 days.

Cell transfection

Cells were incubated with ALB conjugates (see Table 1), Pip6a-PMO prepared using the SELPEPCON method, and Pip6a-PMO and apolipoprotein E (ApoE)-PMO prepared by direct conjugation, which were made in serum-free Opti-MEM, and 200 μL was added to each well as duplicates and incubated at 37°C for 4 hours. The transfection medium was then replaced with DMEM supplemented with 5% horse serum and 1% PSN and the cells incubated for a further 20 hours at 37°C. Cells were washed with PBS and 0.5 mL of TRI RNA (Sigma) isolation reagent was added to each well. Cells were frozen at −80°C for 1 hour.

RNA extraction and nested reverse transcription-polymerase chain reaction analysis

Total cellular RNA was extracted using TRI reagent with an extra further precipitation with ethanol. The purified RNA was quantified using Nanodrop® ND-1000 (Thermo Scientific). The RNA (400 ng) was used as a template for reverse transcription-polymerase chain reaction (RT-PCR) using a OneStep RT-PCR Kit (Roche). The primer sequences used were Exon20F0 5′-CAGAATTCTGCCAATTGCTGAG-3′ and Exon26Ro 5′-TTCTTCAGCTTGTGTCATCC-3′. The cycle conditions for the initial reverse transcription were 50°C for 30 minutes and 94°C for 7 mintes for one cycle followed by 30 cycles of 94°C for 20 secconds, 55°C for 40 seconds, and 68°C for 80 seconds. One microlitre of the RT-PCR product was used as template for the second PCR step. The primer sequences were Exon20Fi 5′-CCCAGTCTACCACCCTATCAGAGC-3′ and Exon2Ri 5′-CCTGCCTTTAAGGCTTCCTT-3′. The amplification was carried out using 0.5 U of SuperTAQ in 25 cycles at 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minutes. the products were separated by electrophoresis using 1.5% agarose gel.

Data analysis

The images of agarose gels were taken on a Molecular Imager ChemiDoc™ XRS+ imaging system (BioRad) and the images were analyzed using Image Lab (V4.1). Microsoft

Table 1. Yields of Parallel Synthesis of the ALB-Peptide Library and ALB-PMOEx23 Conjugate Synthesized by the Synthesis Approach Shown in Fig. 2

Peptide	*Sequence (N- to C-term)*							*Yield peptide*	*Yield conjugate*
ALB1	L	R K L	R K R L	L	R	Bpg		42%	25%
ALB2	R	L L R	K R L K	R	L	Bpg		36%	29%
ALB3	L	K L R	R K R L	L	R	Bpg		32%	20%
ALB4	R	R R R	K K L L	L	L	Bpg		37%	17%
ALB5	L	L L L	K K R R	R	R	Bpg		54%	30%
ALB6	K	L L R	R R R L	L	K	Bpg		45%	22%
ALB7	R	R K L	L L L K	R	R	Bpg		30%	20%
ALB8	L	L K R	R R R K	L	L	Bpg		38%	21%
ALB9	A	R K A	R K R A	A	R	Bpg		55%	45%
ALB10	I	R K I	R K R I	I	R	Bpg		47%	37%
ALB11	F	R K F	R K R F	F	R	Bpg		35%	55%
ALB12	W	R K W	R K R W	W	R	Bpg		52%	29%
ALB13	N	R K N	R K R N	N	R	Bpg		63%	43%
ALB14	H	R K H	R K R H	H	R	Bpg		39%	28%
ALB15	L	R R L	R R R L	L	R	Bpg		41%	56%
ALB16	L	K K L	K K K L	L	K	Bpg		44%	43%

Excel was used to analyze and plot the exon-skipping assay data, which were expressed as the percentage of exon-23 skipping from at least three independent experiments.

Results

Adaptation of SELPEPCON for P-PMO conjugate synthesis

We first applied the principles of SELPEPCON [18] to design chemistry for parallel P-PMO conjugate synthesis. Similar to the synthesis of P-PNA, we chose once again copper-catalysed alkyne-azide ''click'' chemistry for stable conjugation of a peptide library to a PMO cargo (Fig. 2). In adaptation of SELPEPCON to P-PMO synthesis, we had at first utilized a commercially available PMO cargo functionalized at the 3′-terminus with a thiol linker, to which a biotin was subsequently attached, and at the 5′-end with a primary amino group, to which an azido group was subsequently introduced ready for conjugation to the peptide library.

Although this route of SELPEPCON was successful (data not shown), it was more convenient and less expensive to develop methodology that utilized unmodified and unfunctionalized PMO. Therefore, to facilitate the desired cargo functionalization, unmodified PMO was precoupled on its 3′-end to a short connector peptide containing an azido group and a cysteine residue (Fig. 1). This allowed both attachment of a biotin tag through a reversible disulfide linker and click conjugation with an alkyne-containing peptide library. The method then involves rapid purification of the conjugated product via immobilization on a streptavidin cartridge column, washing, release from the support, and desalting. We also report an optional iodoacetamide capping step in order to mask any potential negative influence of the released cysteine thiol group on conjugate activity.

To achieve such syntheses, the PMO component was first coupled to the short connector peptide (Fig. 1). The connector peptide, the sequence of which was chosen to ensure significant serum stability [BWXCG, **1**], was synthesized on solid phase using a Liberty microwave peptide synthesizer. Once the synthesis was complete, 5-azidopentanoic acid was coupled manually to the *N*-terminus of **1** to yield the supported azido connector peptide **2**. The supported azido connector peptide **2** was deprotected and cleaved from the solid support to give the azido peptide **3**. A biotin group was introduced on to **3** by disulfide bridge formation via reaction of the cysteine residue with commercially available *N*-[6-(biotinamido)-hexyl]-3-(2-pyridyldithiopropionamide (EZ-link HPDP biotin, **4**). This reaction provided the N_3-BWXCG-S-S-biotinylated peptide **5** in good yield after HPLC purification. The biotinylated azido connector peptide-PMO conjugate **6** was prepared by reaction of the 3′-end of the PMO with the *C*-terminal carboxylic acid moiety of the peptide to form an amide bond. The PMO used was a 25-mer previously used for exon 23 skipping of the dystrophin gene in mouse *mdx* muscle [16,17].

Peptide and P-PMO library synthesis

For a trial peptide library, we chose a short cationic 10-residue peptide (LRKLRKRLLR, ALB1 in Table 1) ApoE (residues 141–150) as an example. This peptide has been known for some years as a peptide that binds to the LDL receptor [21]. More recently ApoE peptide has been shown, when displayed on nanoliposomes, to have the potential to direct such liposomes and their contents across the blood–brain barrier (BBB) [22]. ApoE peptide and its variants could be of interest as PMO conjugates in respect of whether PMO can be transported across the BBB in addition to enhancing entry into muscle cells.

Peptide variants synthesized in the 16-member library chosen included hydrophobic amino acid substitutions [e.g., leucine (L) to isoleucine (I); L to phenylalanine (F); and L to alanine (A)] as well as rearrangement of the positions of

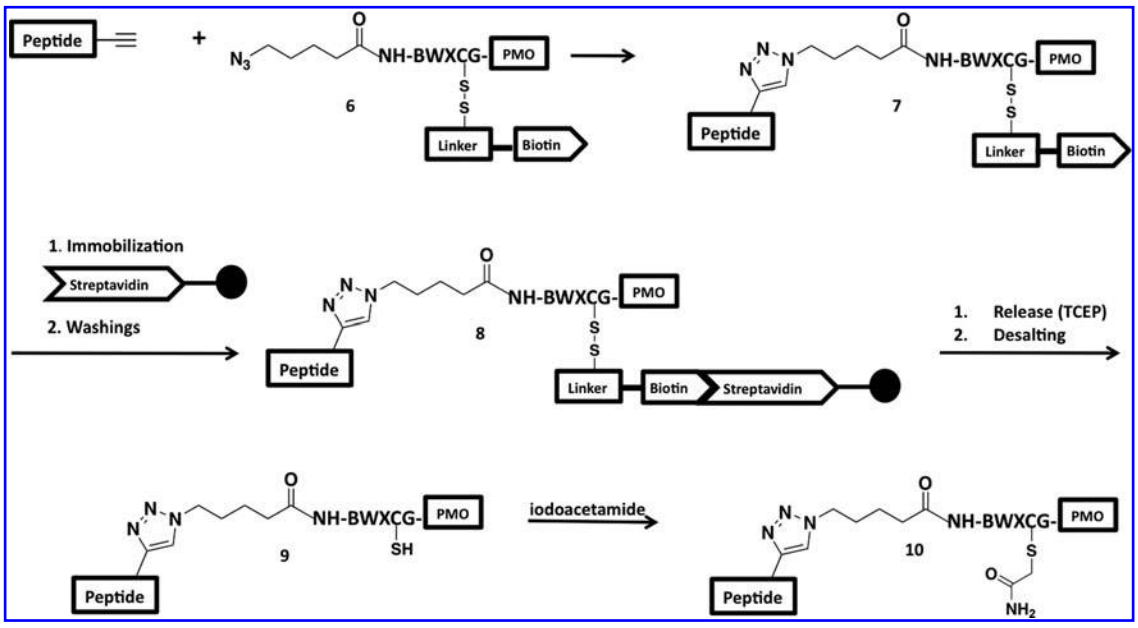

FIG. 2. SELPEPCON peptide (P)-PMO conjugate synthesis by conjugation of a biotinylated azido connector peptide functionalized PMO with a peptide library via alkyne-azide click chemistry followed by purification by streptavidin immobilization and release, and optional cysteine capping using iodoacetamide. TCEP, tris(2-carboxyethyl)phosphine; SELPEPCON, SELection of PEPtide CONjugates.

the cationic residues lysine (K) and arginine (R) (Table 1). For each peptide, a *C*-terminal alkyne was introduced using Fmoc-L-bis-homopropargylglycine (Fmoc-Bpg) through an initial manual coupling to the H-rink amide solid support prior to peptide assembly. The peptide library was synthesized using an Intavis Parallel Peptide Synthesizer by standard Fmoc chemistry [18]. After the final Fmoc deprotection step, the support was *N*-terminally acetylated and the peptides cleaved from the support by standard TFA treatment. The peptides were rapidly isolated by solid phase extraction (SPE) using Oasis HLB cartridges in good yields (Table 1). MALDI-TOF mass spectrometric analysis revealed in all cases that the major product was the expected peptide with only minor amounts of side products present (see Supplementary Table S1 and Supplementary Figs. S1–S9; Supplementary Data are available online at www.liebertpub.com/nat). Cleavage, deprotection, and purification of all 16 peptides were carried out in just one day, thus demonstrating quick and easy parallel synthesis and purification.

Peptide-PMO conjugates were synthesized, purified, and isolated in parallel (Fig. 2). Thus alkyne peptides were conjugated to the biotinylated azido connector peptide-PMO conjugate **6** through copper-mediated alkyne-azide coupling reactions. To stabilize the active copper (I) species, 2,6-lutidine and tris(benzyltriazolylethyl) amine (TBTA) were used [18]. After 2 hours, reactions were quenched by addition of EDTA and the conjugates **(7)** immobilized on a streptavidin-functionalized Sepharose support held in SpinTrap centrifuge tubes (conjugates **8**). Following several washes to remove the excess peptide and reaction reagents, the conjugates **(9)** were released from the support by addition of a solution containing 10 mM (TCEP). Centrifugation led to isolation of the products in the solution, while the biotin tag remained attached to the support. Conjugates were lyophilized and any remaining buffer, and TCEP was removed by solid phase extraction (SPE) using Oasis HLB cartridges in reasonable yields. Representative mass spectra for P-PMO products are shown in Supplementary Figs. S10–S16 and HPLC traces for a representative peptide (ALB1) and its P-PMO conjugate are shown in Supplementary Figs. S17 and S18.

Assay of the ApoE-PMO library in mdx muscle cells and capping

Preliminary analysis of conjugates in an exon-skipping assay in mouse *mdx* muscle cells suggested that significant exon skipping activity was lost for ALB1 compared to a directly conjugated ApoE-PMO conjugate (data not shown). Accordingly, we decided to investigate whether capping of the free thiol functionality of the cysteine in the connector peptide could restore exon-skipping activity. Although the thiol could be successfully capped after the desalting step of the synthesis via reaction with a large excess of iodoacetamide (conjugates **10**, Fig. 2), this required a subsequent extra lyophilization resulting in a slight decrease in overall yield. Therefore, following cleavage from the solid support with TCEP, the resultant solution was treated in situ with a large excess of iodoacetamide. MALDI-TOF analysis showed that capping of the free thiol occurred successfully and thus excess TCEP and iodoacetamide were then removed using the centrifugal filtration units.

The ALB-PMO conjugate library was screened at 1 and 2 μM concentrations for their abilities to induce exon 23 skipping in the well-established skeletal muscle model of DMD (i.e., murine H2k *mdx* cells). In vitro differentiated H2k *mdx* myotubes were treated with ALB-PMO conjugates and exon skipping was evaluated after 24-hour treatment by nested RT-PCR. All conjugates induced exon 23–skipping in a dose dependent manner (Fig. 3). ApoE-PMO prepared using the conventional method of direct conjugation of the *C*-terminal acid of the peptide to the 3′-amine of the PMO via the formation of an amide linkage was used as a positive control in these experiments. Note that the level of exon skipping for ALB1-PMO is only slightly lower than for a control ApoE-PMO where the ApoE peptide is directly conjugated to the PMO. This shows that the additional BWXC(S-cap)G spacer in ALB1-PMO does not reduce the exon skipping activity significantly.

ALB1 to ALB8 all contained the same number of arginine (four), lysine (two), and leucine (four) residues, but placed in different relative positions. It can be seen (Fig. 3) that ALB4, 6, and 8, where all four arginine residues were clustered, gave higher levels of exon skipping than for conjugates where the arginines were not clustered (ALB1, 2, 3, 5, and 7). Interestingly, when all the leucine residues were replaced by alanines and when arginine residues were not clustered (ALB9), this conjugate also showed a higher level of exon skipping than the original ApoE peptide (ALB1). However, replacement of the leucine residues either by isoleucine, phenylalanine, tryptophan, or by asparagine (ALB10–13) resulted in a drop in exon skipping activity. Additionally, replacement of leucine residues by histidines (ALB14) led to higher exon skipping activity compared to the starting ApoE peptide (ALB1). Replacement of all lysine residues by arginines (ALB15) led to an increase in exon skipping. However, replacement of all arginine residues by lysines (ALB16) led to a sharp decrease in the level of exon skipping. This peptide acts essentially as a ''baseline'' for exon skipping activity and also shows that exon skipping is not completely lost in the absence of Arg residues.

These results show the power of the SELPEPCON method in the ability to produce variant peptides with higher levels of biological activity in a cell assay.

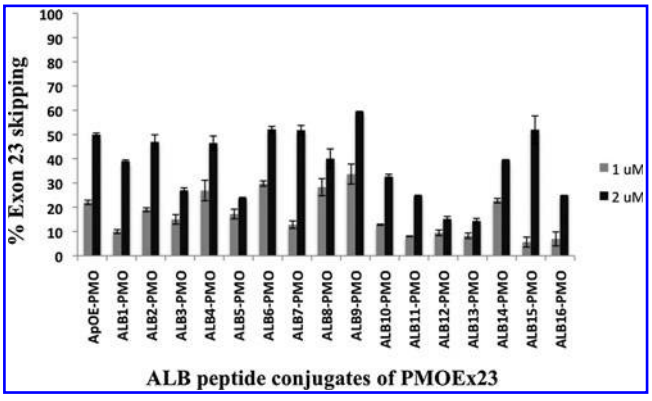

FIG. 3. Analysis of the ALB-PMO conjugate library (Table 1) showing levels of exon 23 skipping in H2k *mdx* muscle cells for the different conjugates. Differentiated H2k *mdx* cells were incubated for 4 hours with conjugates at the indicated concentrations.

An alternative method for parallel P-PMO synthesis

Although the above P-PMO synthesis procedures are suitable in principle for most peptides (other than those containing cysteine, which could perhaps undergo disulfide shuffling during the syntheses), we wished to simplify the conjugation chemistry, especially for arginine-rich peptides, such as Pip6a, which are used most frequently in our laboratory. Rather than initial pre-coupling of a PMO with an azido connector peptide ready for click conjugation, it is possible to dispense with the connector peptide and instead carry out direct amide conjugation of *C*-terminally extended peptide libraries containing a biotin for rapid purification to the PMO.

To exemplify the principle, a single peptide Pip6a was synthesized on solid phase with a *C*-terminal extension of an additional cysteine residue and an XB spacer (Fig. 4) (note that for SELPEPCON, a series of such peptides would be prepared in parallel). Once synthesis was complete the peptide was *N*-acetylated and cleaved from the support. The peptide was biotinylated in a similar fashion to that used previously using EZ-link HPDP Biotin. Conjugation of the peptide to PMO was carried out in solution via *C*-terminal activation of the peptide in the presence of HBTU and HOAt. After 2 hours the reaction was quenched with TBS in CH_3CN and the P-PMO conjugate immobilized as before on a streptavidin-functionalized Sepharose support held in a SpinTrap centrifuge tube. Following several washes, the desired conjugate was released from the solid support using TCEP, and the resultant free thiol was capped by reaction with iodoacetamide and impurities removed by solid-phase extraction using Oasis HLB cartridges (rather than the filtration that was used in the first method) to give the Pip6a-conjugated PMO in good yield. Note that in this method, the excess of biotinylated peptide is also immobilized on the solid support and is released together with conjugate, but such excess peptide is then removed during the final solid-phase extraction.

Comparison of exon skipping levels in mouse *mdx* muscle cells showed that the presence of the extra capped thiol group and XB spacer in the P-PMO prepared using this alternative SELPEPCON method (Pip6a-SCap-PMO) had no significant effect on the exon skipping activity (Supplementary Fig. S19).

Discussion

We have demonstrated convenient and rapid chemical procedures suitable for parallel synthesis of a library of peptide conjugates of PMO via the SELPEPCON approach. In the first method, click conjugation occurs in solution between members of an alkyne-functionalized peptide library with a PMO that is pre-coupled with a biotinylated azido connector peptide. Streptavidin immobilization, reductive release, and capping of the released cysteine residue forms a series of P-PMO conjugates that are pure enough to be used directly in a biological cell assay, in this case using mouse *mdx* cells and screening for exon skipping using an RT-PCR method. It is important to note here that the insertion of a BWXC(S-cap)G spacer between peptides and

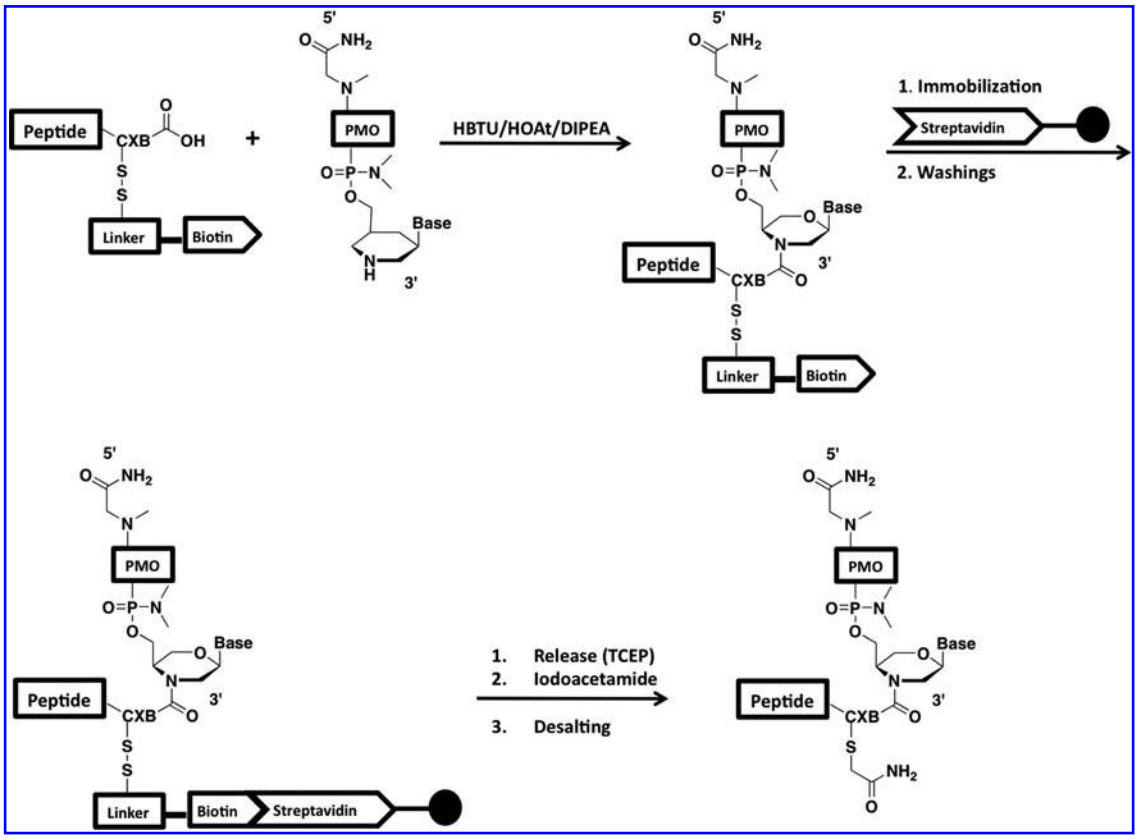

FIG. 4. Alternative route to SELPEPCON P-PMO synthesis, involving synthesis of peptides *C*-terminally extended with C-X-B and subsequently *C*-terminal amide conjugated to a PMO cargo. X, aminohexanoic acid; B, beta-alanine.

PMO cargo did not result in significant loss of exon skipping activity (Fig. 3), nor was any difference seen in exon skipping using a C(S-cap)XB spacer in the second method compared with directly conjugated Pip6a-PMO (Supplementary Fig. S19). In a similar way, conjugation of Pip peptides to either *N*- or *C*-termini of PMO is known to lead to similar levels of exon skipping [23]. All these data reinforce the view that cell-penetrating peptides function predominantly as passive carriers of PMO into the nucleus of cells and therefore do not require specific conjugation methods. However, we do not have an explanation for why a free thiol group in the connector peptide was detrimental to exon skipping in the case of P-PMO (data not shown) but not for splicing redirection of peptide-PNA conjugates in HeLa 705 cells [18].

Whereas the speed and convenience of the synthesis procedures is herein demonstrated, methods of assay in cell culture vary substantially in convenience. We have used exon skipping and RT-PCR analysis for screening P-PMO conjugates which involves use of agarose gels and gel imaging. Such methods are not as rapid, for example, as in the previously reported P-PNA study in HeLa 705 cells, which utilized a luciferase luminescence assay [18]. Hence, we synthesized in this case only a small 16-member P-PMO library. In principle, faster screening assays in primary muscle cells could be developed utilizing mouse cells expressing enhanced green fluorescent protein by splicing redirection using an oligonucleotide [24], which could facilitate muscle cell screening of hundreds of P-PMOs synthesized by the parallel SELPEPCON method.

The ApoE peptide was chosen as a model for this study because it has potential in principle both for development as a drug carrier across the BBB, but also as a cationic peptide that also contains some hydrophobic residues (like Pip peptides [17]), which might be suitable for entry into muscle cells. Thus we found that ALB1-PMO (ApoE peptide conjugated by the SELPEPCON method) does show some exon skipping activity in *mdx* cells. It is not surprising that this is a lot lower than for Pip peptides, partly because there are only four arginine residues in ApoE peptide, whereas Pip peptides, in common with other similarly used arginine-rich peptides, commonly contain 7–10 arginine residues [16,17].

One of the determining features of cell delivery of PMO and PNA by CPPs both in muscle and in HeLa cells is the number of arginine residues [9,18], as also demonstrated in this work when arginine residues were replaced by lysine residues, which led to a significant loss in exon 23 skipping activity (Fig. 3, ALB16-PMO). Furthermore, as seen in Fig. 3, clustering of arginine residues (ALB4–8) increased activity. Replacement of leucine residues by alanine, a smaller hydrophobic amino acid (ALB9), but not by others, also led to higher exon skipping activity in PMO conjugates than the parent ALB1. This is reminiscent of previously published work on the use of nonnatural hydrophobic spacers between arginine residues [25]. Interestingly replacement of leucines by histidines maintained the same exon skipping level as ALB1, which might be as a result of a ''proton sponge effect'' [26]. Such variant ApoE derivatives synthesized could form starting points for further peptide design and testing in a cell or in animal models.

The second P-PMO conjugation approach does not involve use of click chemistry but instead utilizes amide conjugation of members of a peptide library containing a short tripeptide 3′-extension sequence. A single example was shown involving conjugation of the CPP Pip 6a to PMO. This method is particularly suitable for peptides such as Pip6a that contain no lysine residues, which we anticipate investigating more extensively in forthcoming P-PMO studies of neuromuscular diseases, such as DMD and spinal muscular atrophy. However, it should be noted that lysine-containing peptides can be accommodated by use of orthologous 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl protection, as we described recently for synthesis of fluorescently labeled P-PMO [27]. This second method also involves fewer steps in synthesis, since there is no requirement for separate connector peptide synthesis, but this must be balanced against the need for an extra deprotection step after conjugation should lysine residues be used in the peptides, and also the fact that excess peptides are only removed during final solid-phase extraction, rather than during the washings following immobilization.

In summary, we expect the methods of parallel peptide-PMO conjugate synthesis described to substantially improve the rate of search of peptide chemical space for desired activity parameters in cell culture studies and hence to give rise to an increasing number of candidates for therapeutic studies of P-PMO.

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Author Disclosure Statement

A patent describing this work has been applied for and has been licensed to Cambridge Research Biochemicals, Billingham, United Kingdom. No other competing financial interests exist.

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